

Developmental changes in amniotic and allantoic fluid insulin-like growth factor (IGF)-I and -II concentrations of avian embryos

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Abstract

In the literature, IGFs in the developing embryo are usually determined by blood serum concentrations. For this study, IGF-I/-II was quantified in the amniotic and allantoic fluids of fertile commercial broiler chicken (*Gallus domesticus*) ($n=222$), Pekin duck (*Anas platyrhynchos*) ($n=250$), and turkey (*Meleagris gallopavo*) eggs ($n=200$) during incubation. Amniotic and allantoic fluids were collected from embryos starting at 6 days of incubation for chickens and 8 days of incubation for ducks and turkeys. IGF concentrations within the fluids were determined by radioimmunoassay. Chicken amniotic IGF-I concentration at stage 29 of development was significantly higher ($P \leq 0.05$) than the duck or turkey. At stage 36 of development the concentration of IGF-II in the amniotic fluid was 2.8 times greater in the chicken versus the duck ($P \leq 0.05$) and 2 times greater than in the turkey ($P \leq 0.05$). Within species, chicken IGF-I concentration in the amniotic fluid had a cubic trend ($P \leq 0.001$), duck IGF-I increased linearly ($P \leq 0.001$), and turkey concentrations declined quadratically ($P \leq 0.001$) throughout development. In all species, the IGF-II concentration was higher than the IGF-I concentration in the amniotic and allantoic fluids.

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1. Introduction

Somatomedins exert potent anabolic and mitogenic effects on cells. Two of the most studied somatomedins are C and A which have come to be known as insulin-like growth factors (IGF)-I and -II, respectively. Collectively the IGFs act via endocrine or paracrine mechanisms and in mammals are known as the major mediators for growth hormone (GH).

IGF-I and IGF-II in chickens vary by eight AA for IGF-I and twelve for IGF-II from human (Rinderknecht and Humbel, 1978a,b), bovine (Francis et al., 1988) and porcine (Francis et al., 1989), respectively (McMurtry et al., 1997, 1998). Duck and turkey IGF-I do not vary from that of the chicken with regard to AA composition (Kansaku et al., 2003; Czerwinski et al., 1998). No differences in IGF-II exist between turkey and chicken (Richards et al., 2005).

Although receptor binding is not the focus of this paper, the avian and mammalian species differ in IGF receptors. The avian species has both receptors, IGF-IR and IGF-IIR/M6P-R, present. However, IGF-I and -II both bind to a common receptor, IGF-IR (McMurtry et al., 1997). The chicken IGF-IR is 85% identical to human (Holzenberger et al., 1996) and the IGF-IIR is 60% identical to human and bovine (Zhou et al., 1995).

While there may be differences in the AA composition of IGFs and their receptors between mammalian and avian species, the effects elicited by their presence are homologous. IGF-I plays a prominent role in adult life by influencing the metabolic, growth, and differentiation processes that occur within the body of the growing organism. IGF-II appears to influence the fetal organism by stimulating undifferentiated cells to differentiate (Heyner et al., 1990). Therefore, research suggests IGF-II is more influential in embryonic growth and development, whereas IGF-I plays a larger role in neonatal and adult growth (De Pablo et al., 1991; Heyner et al., 1990; Sussenbach et al., 1991).

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The extra-embryonic sac that surrounds the embryo, the amnion, contains fluid to protect the embryo from infection, mechanical injury, desiccation, and adhesion (Romanoff and Romanoff, 1967). The chick begins imbibing the amniotic fluid around day 13 of incubation and continues until internal pipping (day 19 of incubation). Hence, the embryo is exposed to and swallows a fluid containing proteins, minerals, water, hormones, and any other nutrients needed for growth and development. The same is true in developing fetuses of mammals. Harding et al. (1984) and Trahair et al. (1986) evaluated the impact of fetal swallowing in sheep fetuses and found it is important for proper gastrointestinal tract (GIT) development. These findings suggest IGF-I may play a major role in proper GIT development of mammals prior to parturition.

Fluctuating concentrations of IGF-I/-II in the developing embryo have been reported in the literature; however, the majority of the data documents what occurs within the blood serum and not necessarily within the amniotic and allantoic fluids that surround the developing embryo. Recently, Applegate et al. (2005) reported accelerated villus growth in the duck jejunum compared to turkey poults following hatch; which is notable given that both species hatch from similar sized eggs and have similar incubation periods. The dramatic change in villus growth following hatch may be attributed to diet consumption, digestive enzyme activity, and nutrient transporter numbers (Applegate et al., 2005). Significant differences with ducks having longer villi and deeper crypts at day of hatch suggest a possible hormonal influence on GIT development in the egg. The reported impact on GIT development due to IGF in mammals, may potentially explain the observations by Applegate et al. (2005). Therefore, the intent of this project was to quantify the presence and abundance of IGF-I/-II within the amniotic and allantoic fluids during development of the chicken (*Gallus domesticus*), duck (*Anas platyrhynchos*), and turkey (*Meleagris gallopavo*).

2. Materials and methods

Fertile commercial broiler eggs ($n = 222$), Pekin duck eggs ($n = 250$), and turkey eggs ($n = 200$) were obtained and incubated separately in Jamesway¹ incubators. Amniotic and allantoic fluids were collected from embryos starting at 6 days of incubation for chickens and 8 days of incubation for ducks and turkeys to normalize by stage of embryonic development (Table 1). The embryos were broken into a petri dish with all membranes intact and allantoic fluid was collected using a 1 cc syringe with a 23 gauge needle. After the fluid was collected, the allantoic sac was dissected away and the amniotic fluid was collected. After the initial collection, fluids (amniotic and allantoic) were collected every other day with 8 eggs collected per species per day.

Embryos were classified by stage of development based upon Hamburger and Hamilton (1951). While Hamburger and

Table 1

Relationship between stage of development and day of incubation

Stage	Species		
	Chicken	Duck	Turkey
	(Day of incubation)		
29	6	8	8
33	8	10	10
36	10	12	12
37		14	14
38	12	16	16
39		18	18
40	14	20	20
41		22	22
42	16	24	24
43			26
44	18		

Hamilton (1951) classified stages of development only for chickens, the initial collection day for the duck and turkey was established using the following mathematical equation ($d = (28/21) * 6$) where 6 is the first collection day of the chicken. Once the relative incubation day was determined for the duck and turkey, the morphological characteristics of development for the chicken were used for determining the stage of development for the duck and turkey. While using stage of development for each species allowed easier inter-species comparison, Table 1 relates stage of development to day of incubation for quicker reference. Amniotic and allantoic fluid samples were frozen and stored at -80°C prior to analysis.

The concentrations of IGF-I and IGF-II were determined using radioimmunoassay (RIA) as described by McMurtry et al. (1994, 1998). Due to the quantity collected for the amniotic and allantoic samples, the IGF-II analysis required some samples within species and stage to be pooled. Therefore, some replicates within species and stage were pooled. In Tables 2 and 3, the superscript 'p' denotes those samples where at least 2 replicates were pooled for analysis. Variation in n reported in Tables 2 and 3 occurred for two reasons. First, a limited number of eggs were set, so if a fluid was not sampled no extra eggs were used to obtain the missing sample for that day. Secondly, data points were removed following RIA analysis due to high C.V. or being a statistical outlier (2 standard deviations beyond the mean).

The RIA intra-assay variation for IGF-I was 4.6% and 3.9% for IGF-II with no inter-assay variation. The data were analyzed across species utilizing the General Linear Model (GLM) procedure of SAS (SAS Institute, Cary, NC) with least-squares means separations using Tukey correction for multiple means comparisons. Significance for mean differences was set at $P < 0.05$. Data were also analyzed within species using the same procedure with orthogonal contrasts testing for a linear, quadratic, or cubic response during development.

3. Results

The concentrations of IGF-I/-II can be found in Tables 2 and 3 for amniotic and allantoic fluids, respectively. Across species, IGF-I was significantly different only in the amniotic fluid.

¹ Jamesway Incubator Company Inc., Cambridge, Ontario, Canada.

Table 2

Amniotic fluid insulin-like growth factor (IGF)-I and -II concentrations in chickens, Pekin ducks, and turkeys during incubation

Stage	Species								
	Chicken			Duck			Turkey		
	(IGF-I, ng/ml)								
	Mean	S.D.	<i>n</i>	Mean	S.D.	<i>n</i>	Mean	S.D.	<i>n</i>
29	0.987 ^y	0.625	7	0.182 ^x	0.070	4	0.020 ^x	0.000	3
33	0.066	0.062	4	0.066	0.074	8	0.041	0.042	4
36	0.209	0.168	5	0.128	0.114	3	0.514	0.432	7
37				0.259 ^x	0.302	6	1.288 ^y	0.457	3
38	0.504 ^x	0.367	7	0.442 ^x	0.301	7	1.729 ^y	0.535	6
39				1.041	0.396	6	1.422	0.602	6
40	1.378	0.378	8	0.900	0.302	8	1.136	1.041	8
41				0.539	0.132	8	0.853	0.565	9
42	1.216	1.141	7	1.198	0.318	5	0.913	0.528	8
43							0.789	0.347	2
44	1.026	0.331	8						

Source of variation	Probability		
Linear	0.005	0.001	0.024
Quadratic	NS	NS	0.001
Cubic	0.001	NS	NS

(IGF-II, ng/ml)									
29	1.878	—	1 ^p	1.424	—	1 ^p			
33	3.063	2.757	4 ^p	1.246	0.865	6	0.560	0.000	4
36	3.154 ^y	0.820	6	1.130 ^x	0.542	7	1.587 ^{xy}	1.240	5
37				1.718	0.830	5	1.175	1.150	5
38	5.168	1.648	6	1.296	1.499	7	1.694	1.427	7
39				0.560	0.000	8 ^p	0.560	0.000	6
40	2.255	2.170	7	0.560	0.000	8	1.331	1.463	7 ^p
41				0.931	0.720	8	0.576	0.042	7 ^p
42	0.561	0.122	4 ^p	4.819	1.520	2 ^p	0.576	0.042	7
43							2.070	—	1 ^p
44	2.421	2.239	7 ^p						

Source of variation	Probability		
Linear	NS	0.019	NS
Quadratic	NS	0.001	NS
Cubic	NS	0.001	0.059

NS, probability of stage effect was not significant ($P \geq 0.05$).

^{x-y} Means within rows with common superscript are not significantly different ($P \geq 0.05$ using Tukey multiple means comparison).

^p Pooled samples.

Chicken amniotic IGF-I concentration at stage 29 of development (wing bent at the elbow on the embryo and presence of a prominent beak) was significantly higher ($P \leq 0.05$) than in the duck or turkey. At stage 37 of development, the turkey concentration was significantly higher than in the duck ($P \leq 0.05$). The outline of the developing scales covering the entire leg is a defining characteristic for stage 38. At this point in development, the turkey IGF-I concentration was higher than the chicken or duck ($P \leq 0.05$). Throughout the rest of the incubation period, no differences in IGF-I concentration were detected across the three species in either the amniotic or allantoic fluids.

On the other hand, IGF-II concentrations differed in both the amniotic and allantoic fluids across species. At stage 36 of

development (tapering of the claws and the lower lid of the eye grown upward to be level with the cornea) the concentration of IGF-II in the amniotic fluid was 2.8 times greater in the chicken than in the duck ($P \leq 0.05$) and 2 times greater than in the turkey ($P \leq 0.05$). Within the allantoic fluid, IGF-II was considerably higher in the turkey than in the duck ($P \leq 0.05$) at stage 41 of incubation (the embryo is entirely formed, and stage of development is determined by length of beak and middle toe (Hamburger and Hamilton, 1951)). While differences were seen across species, fluctuating concentrations were also observed within species throughout incubation.

The chicken IGF-I concentration in the amniotic fluid is best described with a cubic curve ($P \leq 0.001$). The cubic trend had a minimum concentration at stage 33 of development and a

Table 3

Allantoic fluid insulin-like growth factor (IGF)-I and -II concentrations in chickens, Pekin ducks, and turkeys during incubation

Stage	Species								
	Chicken			Duck			Turkey		
	(IGF-I, ng/ml)								
	Mean	S.D.	<i>n</i>	Mean	S.D.	<i>n</i>	Mean	S.D.	<i>n</i>
29	0.605	0.408	5	0.194	0.149	4	0.160	0.082	7
33	0.009	—	1	0.049	0.076	7	0.133	0.076	4
36	0.045	0.034	5	0.112	0.080	3	0.151	0.071	6
37				0.194	0.079	2	0.411	0.028	2
38	0.415	0.548	2				0.346	0.319	2
39				0.515	—	1	0.137	—	1
40				0.556	0.450	4	0.053	0.028	2
41				0.256	—	1			
42				0.318	0.465	3	0.304	0.092	4
43							0.502	—	1
44	0.020	—	1						

Source of variation	Probability		
Linear	NS	NS	0.015
Quadratic	NS	NS	NS
Cubic	NS	NS	0.004

(IGF-II, ng/ml)									
29	5.118	—	1 ^p	3.918	1.653	6 ^p	3.388	1.147	4 ^p
33	2.598	—	1 ^p	2.208	0.365	4 ^p	2.181	0.821	7
36	2.491	0.745	5	2.178	1.435	4	1.445	0.776	9
37				1.151	0.156	2	0.840	0.626	5 ^p
38	3.010	2.130	3	0.560	—	1	1.112	—	1
39							0.560	0.000	2
40	1.606	1.597	5	0.746	0.416	5	3.010	2.166	3
41				1.958 ^x	1.977	2	8.213 ^y	—	1
42				2.582	1.760	3	2.005	2.889	4
43							0.560	—	1
44									

Source of variation	Probability		
Linear	NS	NS	NS
Quadratic	NS	0.006	NS
Cubic	NS	NS	0.001

NS, probability of stage effect was not significant ($P \geq 0.05$).

^{x-y} Means within rows with common superscript are not significantly different ($P \geq 0.05$ using Tukey multiple means comparison).

^p Pooled samples.

maximum concentration at stage 40 of development. Contrastingly, the allantoic fluid IGF-I concentration could not be explained by a linear, quadratic, or cubic curve throughout incubation. During the majority of the incubation period the IGF-I in the allantoic fluid was lower than the IGF-I present in the amniotic fluid.

The duck IGF-I concentration in the amniotic fluid followed a positive linear curve ($P \leq 0.001$) throughout development. The lowest concentration was observed at stage 33 of development with the maximum concentration measured at stage 42 of development.

Similar to the observation of IGF-I in the allantoic fluid of the chicken, no significant response was noted with IGF-I in the allantoic fluid of the duck. The concentration of IGF-I in the allantoic fluid was similar to the amniotic fluid until stage 37. At stage 37 of development, the IGF-I concentration in the amniotic fluid doubled compared to the allantoic fluid. The trend of the IGF-I concentration in the amniotic fluid was observed throughout the rest of incubation.

Turkey IGF-I concentrations were very intriguing, in that concentrations in amniotic fluid followed a quadratic trend ($P \leq 0.001$) with the maximum concentration observed at stage 38 of development. Unlike observations in the allantoic fluids of the chicken and duck, a cubic response ($P \leq 0.004$) was observed in the allantoic fluid throughout development. The lowest amount measured was stage 40 and the maximum at stage 43 of development just prior to internal pipping. The allantoic concentration of IGF-I was higher than concentrations observed in the amniotic fluid until stage 36. At this point, the IGF-I concentration in the amniotic fluid became approximately 3.5 times higher than the IGF-I concentration in the allantoic fluid.

In all species, the IGF-II concentration was higher than the IGF-I concentration in the amniotic and allantoic fluids. The chicken IGF-II concentration was greater in the amniotic fluid than the allantoic fluid from stage 33 to hatch with no linear, quadratic, or cubic trend evident throughout the incubation period.

On the other hand, the duck IGF-I, in the amniotic fluid, could be explained by a cubic curve ($P \leq 0.001$) with the lowest concentration at stage 39 and 40 of development and a maximum at stage 42 of development. The IGF-II in the allantoic fluid followed a quadratic curve ($P \leq 0.006$) with the lowest concentration noted at stage 38 of development.

The turkey IGF-II concentrations in both the amniotic ($P \leq 0.059$) and allantoic ($P \leq 0.001$) fluids could be best explained by a cubic curve. The cubic curve within the amnion had a maximum at stage 43 of development. A maximum IGF-II concentration in the allantoic fluid was noted at stage 40 of development.

4. Discussion

The maternal influence in avian species differs dramatically from mammalian species. While mammals can influence the development of the fetus both during development and after parturition, birds' maternal influence is manifested in the

composition of the egg. All necessary nutritional elements, hormones, and molecular machinery needed for development and growth must be present within the yolk, albumen, and shell. IGF-I has been reported in the yolk of unfertilized White Leghorn hens from 0.18 – 0.8 ng/g of yolk (Scavo et al., 1989). As early as stage 4 of development, the embryo begins producing IGF-I/-II as was noted by Allan et al. (2003) through detection of mRNA expression for IGF-I/-II in Hensen's Node. The presence of mRNA for both the receptor and hormone supports IGF acting in a paracrine/autocrine manner because the endocrine system is not yet developed at that stage of development (Allan et al., 2003).

Avian embryos develop in a cleidoic system where all resources necessary for proper growth and development must be present. As development proceeds, restructuring of tissues by the embryo may be catabolized to basic components consisting of AA, glucose, and other cellular metabolites. These components may be stored and later recycled to create new proteins, enzymes, etc. instead of being broken into waste products. Past perception was that the allantoic sac was thought of as repository for waste from the developing embryo (Romanoff and Romanoff, 1967). However, ten Busch et al. (1997) conducted a series of experiments exploring the possibility of the allantois as a storage area. Consequently, the allantois may be a molecular or metabolite storage area as well as a membrane needed for the collection of embryonic waste products (ten Busch et al., 1997).

ten Busch et al. (1997) concluded that three barriers exist in the developing embryo: blood/amnion, blood/allantois, and allantois/amnion. ten Busch et al. (1997) evaluated over 40 amino acids and related amino compounds (base molecule of compound a derivative of an amino acid) and found 39 of them present in the allantois while only 32 could be found in the amnion. For example, the arginine concentration was highest in the allantoic fluid while GABA could not be detected in the amniotic fluid. These AA and related compounds may be regulated by the three barriers present during incubation. Hohlweg et al. (1999) hypothesized the barriers may be controlled hormonally and using insulin and prolactin evaluated their specific influence on the movement of specific amino compounds amongst the three embryonic fluids. A HPLC-fluorometric technique was used to evaluate changes 30 min after the hormone, insulin or prolactin, was administered to the chorioallantoic membrane. The observed changes of AA concentrations in the three fluids indicate a hormonal control. The changes between the three fluids suggests the barriers (blood/amnion, blood/allantois, and allantois/amnion) are bidirectional and could be seen as six 'sub-barriers' (Piechotta et al., 1998). Schmidek et al. (2001) evaluated the effects on amino acid concentrations in plasma, amniotic, and allantoic fluid when IGF-I was placed on the chorioallantoic membrane of the developing embryo. Several of the proteinogenic amino acids (Met, Thr, Leu, etc.) in the amniotic fluid increased while the same amino acids decreased in the plasma at the same time. Although Schmidek et al. (2001) applied approximately 6 times the IGF-I concentration found in the amniotic fluid, the role IGF-I may play on un-innervated membranes within the

egg during development is unknown. However, hormonal control of the barrier would allow the developing embryo to more efficiently transport amino acids into the amniotic fluid. Amino acids could be used as metabolites to nourish the developing embryo as well as providing the necessary building blocks for proteins. If AA can pass relatively easily through the 'sub-barriers' as defined above, it could also be possible for smaller peptides such as IGF-I/-II to be moved easily. With this thought in mind, the decreasing IGF-I in the allantois coinciding with the peak of IGF-I in the amnion may not be just a coincidence and warrants further study.

Circulating IGF-I/-II concentrations in embryos have been documented, as well as studies evaluating the effects of age, nutrition, and growth of post-hatch birds (for review, see [McMurtry et al., 1997](#)). Embryonic chicken IGF-I serum concentrations have been reported with a range of 4–50 ng/mL ([De Pablo et al., 1991](#); [Robcis et al., 1991](#); [Kikuchi et al., 1991](#); [Scanes et al., 1997](#)). The data from these studies follow a similar trend of increasing to 15 days of incubation, peaking, and then decreasing to day of hatch.

On the other hand, reports of circulating IGF-II concentrations in the chicken are conflicting. [McMurtry et al. \(1998\)](#) noted that IGF-II increased (100–135 ng/mL) during the incubation period while [Scanes et al. \(1997\)](#) reported that IGF-II decreased (28–15 ng/mL). The different reports may reflect the differences between Leghorn ([Scanes et al., 1997](#)) and broiler chicken ([McMurtry et al., 1998](#)) embryos. [Scanes et al. \(1997\)](#) reported concentrations of IGF-I and -II to be fairly similar (IGF-I: 18–45 ng/mL; IGF-II: 15–28 ng/mL). In contrast, [McMurtry et al. \(1998\)](#) found IGF-II concentrations to be approximately 10-fold higher than IGF-I in chicken serum.

IGF-I/-II concentrations have also been evaluated in the developing turkey embryo. [McMurtry et al. \(1994\)](#) reported IGF-I in turkey serum (20–45 ng/mL) with a peak at 16 days of incubation. A later study by [McMurtry et al. \(1998\)](#) noted that the peak (considerably lower at 5–25 ng/mL) did not occur until around 24 days of incubation, with a decrease to less than 10 ng/mL prior to hatch. IGF-II peaked at 20–90 ng/mL at around day 26 and decreased to less than 40 ng/mL prior to hatch. When comparing the trends, IGF-II was significantly higher than IGF-I throughout development.

The IGF-I concentration in amniotic fluid was higher than the allantoic fluid for all three species ([Table 2](#)). The peak observed at day 14 in the chicken may reflect the IGF-I peak at day 15 reported in blood serum ([De Pablo et al., 1991](#); [Robcis et al., 1991](#); [Kikuchi et al., 1991](#); [Scanes et al., 1997](#)). The peak at day 16 of incubation in the turkeys' amniotic fluid corresponds to a peak seen in IGF-I in developing turkeys' serum at the same time ([McMurtry et al., 1996](#)). Using in ovo (incubated in the shell) and ex ovo (incubated under shell-less conditions) turkey embryos, [McMurtry et al. \(1996\)](#) observed that ex ovo turkey embryos had no spike in the IGF-I at day 16 of incubation. Body weights between in ovo and ex ovo embryos became significantly different from each other at day 16 and continued through day 25 of incubation ([McMurtry et al., 1996](#)). Therefore, natural development of the embryo depends upon the IGF-I spike on day 16. The lower body

weight and lack of an IGF-I spike at day 16 suggests IGF-I triggers or plays a significant role in development of the embryo. The duck IGF-I concentration in the amnion had a slight peak at day 18 of incubation, however, the IGF-I concentration continues to increase throughout development. The absence of any literature which deals with the IGF-I concentration in serum from duck embryos makes it difficult to suggest if a similar pattern would be expected in the serum.

The IGF-II concentrations observed in the amnion and allantois for the chicken, duck, and turkey are relatively the same within species ([Tables 2 and 3](#)). Data in [Table 3](#) suggest the turkey allantoic fluid had a spike at day 22. However, due to a single embryo being sampled, the data point is more likely a biological anomaly than the biological norm. The duck IGF-II concentration in the amnion changes drastically the last few days prior to hatch. Further investigation is needed to ascertain whether this pattern is different compared to the chick and turkey embryos.

In summary: the IGF-I concentrations observed in the amniotic fluid appear to follow the same progression of increasing, peaking, and decreasing throughout incubation as seen in serum. On the other hand, IGF-II concentrations are fairly consistent during incubation and are relatively higher in the amnion and allantois compared to the IGF-I concentrations during development. Differences observed between IGF-I/-II in the amniotic fluid throughout development both within and across species warrant further studies into the kinetics of IGF-receptor interactions and future exploration into the biological differences in embryonic development between species.

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